APPENDIX II

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High Prevalence and Possible *de Novo* Formation of *BRAF* Mutation in Metastasized Papillary Thyroid Cancer in Lymph Nodes

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Context: The role of the T1799A *BRAF* mutation in lymph node metastasis of papillary thyroid cancer (PTC) is not clear.

Objective: Our objective was to explore the relationship between *BRAF* mutation and lymph node metastasis of PTC by examining the mutation in both the primary tumors and their paired lymph node metastases.

Design: We isolated genomic DNA from primary thyroid tumors and paired lymph node metastases and performed direct sequencing of exon 15 of the *BRAF* gene mutation that carries the T1799A mutation.

Results: In a series of 33 cases, 21 harbored the T1799A mutation in the primary tumors, and 17 (81%) of them harbored the same mutation also in the paired lymph node metastases. Twelve cases did not harbor the T1799A mutation in the primary tumors, among which nine cases also did not harbor *BRAF* mutation in the lymph node-metastasized tumors, whereas the other three did harbor the T1799A mutation in lymph node-metastasized tumor tissues. A novel tandem TG1799–1800AA mutation within one allele was found in a lymph node-metastasized tumor but not in the primary tumor. This mutation results in the change of codon 600 (GTG) of the gene to GAA with the consequent amino acid change (V600E) in the B-type Raf (BRAF) protein, same as that caused by the T1799A mutation alone.

Conclusion: The high prevalence of *BRAF* mutation in lymph node-metastasized PTC tissues from *BRAF* mutation-positive primary tumors and the possible *de novo* formation of *BRAF* mutation in lymph node-metastasized PTC were consistent with a role of *BRAF* mutation in facilitating the metastasis and progression of PTC in lymph nodes. (*J Clin Endocrinol Metab* 90: 5265–5269, 2005)

BRAF MUTATION IS common in human cancers, with a prevalence of up to 66–83% in melanoma and thyroid cancer (1, 2). The most common *BRAF* mutation is the missense T1799A transversion mutation formerly called T1796A mutation (3). Raf kinase is a key component of the RAS→RAF→MAPK kinase→ERK/MAPK signaling pathway, which plays a fundamental role in the regulation of cell growth, division, and proliferation (4). The B-type Raf (BRAF) is the strongest activator of the downstream MAPK signaling in many cells (5). The T1799A mutation causes a V600E (formerly V599E) amino acid change in BRAF protein, conferring the kinase oncogenic function through constitutive activation of the MAPK signaling pathway (1). The T1799A *BRAF* mutation found in thyroid cancer occurs exclusively in papillary thyroid cancer (PTC) and some PTC-derived anaplastic thyroid cancers (6–13).

PTC is the most common thyroid cancer, accounting for 80% or more of all thyroid cancers (14). Although this cancer is usually curable with standard surgical and medical treatments, many patients do have recurrence (15–17). Recurrence of PTC most commonly occurs in local lymph nodes in the neck (18–20), consistent with the general finding that, among different types of thyroid cancers, PTC is most commonly associated with local lymph node metastasis at the initial surgery (21, 22). Recently, the T1799A *BRAF* mutation in primary PTC tumor was found to be associated with a significantly higher prevalence of lymph node metastasis, distant metastasis, and extra-thyroidal invasion in some studies (2, 9, 10). These data suggest that *BRAF* mutation may play an important role in the progression and recurrence of PTC. However, other studies, albeit smaller, showed no statistically significant correlation of this mutation with tumor metastasis, although a tendency of such a correlation was observed in some of them (8, 12, 13). To explore further the role of *BRAF* mutation in thyroid cancer metastasis, in the present study we examined and compared the status of *BRAF* mutation in a series of primary PTC tumors and their paired lymph node-metastasized tumors.

Patients and Methods

Tumor tissues and DNA isolation

This study was conducted based on protocols approved by our institutional review boards. Tumor specimens were obtained from 33 patients who underwent surgery from 1999–2004 at the Center for Endocrine Surgery, Kiev, Ukraine. Among these patients, four were younger than 20 yr old, two were not born yet, and the remaining 27 patients were adults in 1986 when the Chernobyl nuclear accident occurred. Their clinicopathological characteristics are summarized in Ta-

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Abbreviations: BRAF, B-type Raf; PTC, papillary thyroid cancer.

ble 1. All the primary tumors and matched neck lymph node-metastasized tumor samples were paraffin embedded. All tumor samples were examined carefully, and histological diagnoses were established according to the World Health Organization classification by two pathologists who specialize in thyroid pathology (V.V. and V.S.). All primary tumors included in this study were classified as classical variants of PTC; tumors with morphological features of follicular variant of PTC were not included in the present study.

For genomic DNA extraction, 5- and 10-μm-thick serial sections were made for each specimen. The 5-μm sections were stained with hematoxylin and eosin and examined to confirm the histological diagnosis. The 10 μm-thick sections were used for DNA extraction. The number of 10-μm-thick sections available for DNA extraction ranged from one to five. Any tissue surrounding the tumor including normal thyroid, connective tissue, inflammatory cells, and necrotic or hemorrhagic zones was carefully pared away using a scalpel under microscopic observation. The purpose of paring was to ensure that tumor cells composed over 90% of the remaining specimen. Microdissected tumor tissues from paraffin-embedded primary tumors and their matched lymph nodes-metastasized tumors were subjected to treatment with xylene for 8 h at room temperature to remove the paraffin, followed by digestion with 1% SDS and 0.5 mg/ml proteinase K at 48 C for 48 h, with two interval additions of a spiking dose of concentrated proteinase K. DNA was subsequently isolated from the digested tissues with standard phenol-chloroform extraction procedures and precipitated with 70% ethanol and resuspended in water.

BRAF mutation analysis

Because the T1799A transverse mutation in exon 15 of the *BRAF* gene is exclusively found in PTC with a high prevalence, we analyzed this mutation on our tumor samples by direct DNA sequencing as previously described (11). Briefly, exon 15 containing the site where *T1796A* mutation occurs was amplified with PCR using primers TCATAATGCTTGCTCTGATAGGA (forward) and GGCCAAAAATTTAATCAGTGGA (reverse). The PCR was run with a step-down protocol: 95 C for 5 min for one cycle; 95 C for 1 min, 60 C for 1 min, and 72 C for 1 min for two cycles; 95 C for 1 min, 58 C for 1 min, and 72 C for 1 min for two cycles; and 95 C for 1 min, 56 C for 1 min, and 72 C for 1 min for 40 cycles. This was followed by a final extension at 72 C for 5 min. The 30-μl final reaction mixture contained 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 10 mM 2-mercaptoethanol, 1.5 mM each deoxynucleotide triphosphate, 6.7 mM $MgCl_2$, 5% dimethylsulfoxide, 1.67 μM each primers (forward and reverse), 60 ng genomic DNA, and 0.5 U of platinum DNA *Taq* polymerase (Life Technologies, Inc., Gaithersburg, MD). A single band of PCR product with expected molecular weight was confirmed on a 1.5% agarose gel. The PCR products were then subjected to sequencing reaction using the forward primer described above and Big Dye terminator V 3.0 cycle sequencing reagents (Applied Biosystems, Foster City, CA), and the reaction was run on a PCR cycler: 95 C for 30 sec for one cycle and 95 C for 15 sec, 50 C for 15 sec, and 60 C for 4 min for 35 cycles. DNA sequence was then read on an ABI PRISM 3700 DNA analyzer (Applied Biosystems), and the *BRAF* mutations were identified. For case 4, the PCR products of *BRAF* exon 15 were also subcloned, followed by direct DNA sequencing to confirm a tandem *BRAF* mutation.

Results

We analyzed 33 cases of conventional PTC for which we had available both the primary tumors and the matched neck lymph node-metastasized cancer tissues. The overall prev-

TABLE 1. Demographical characteristics and *BRAF* mutation status in primary PTC and paired lymph node metastases

Case no.	Gender	Age at diagnosis (yr)	Age in 1986 (yr)	Tumor size (mm)	Multifocality of the tumor[a]	T1799A *BRAF* mutation	
						Primary tumor	Metastasis
1	F	40	25	30	Yes	Yes	Yes
2	F	34	17	22	Yes	Yes	Yes
3	F	16	−2	30	No	No	No
4	F	59	44	30	No	No	Yes (also G1800A)
5	F	40	25	20	Yes	Yes	Yes (two nodes)
6	F	35	20	11	No	Yes	Yes
7	F	40	25	15	Yes	Yes	Yes
8	F	38	23	15	Yes	Yes	Yes
9	F	46	32	20	No	Yes	Yes
10	F	46	31	23	Yes	Yes	Yes
11	F	39	24	17	No	Yes	Yes
12	F	39	24	30	No	Yes	Yes
13	F	19	4	15	No	Yes	Yes
14	F	34	20	25	Yes	Yes	Yes
15	F	36	22	25	No	Yes	Yes
16	M	14	−2	21	No	No	Yes
17	F	60	46	15	Yes	Yes	No
18	M	20	7	20	No	No	No
19	F	68	54	25	Yes	Yes	No
20	M	40	27	20	Yes	Yes	No
21	M	43	30	15	Yes	No	No
22	M	63	50	70	No	Yes	Yes
23	M	26	13	40	No	No	No
24	M	50	35	40	Yes	Yes	No
25	F	33	24	15	No	No	Yes
26	F	71	53	23	Yes	No	No
27	F	65	47	23	Yes	No	No
28	F	40	22	15	Yes	No	No
29	F	46	28	20	Yes	Yes	Yes
30	M	43	26	27	No	Yes	Yes
31	M	49	31	40	Yes	Yes	Yes
32	F	45	28	35	Yes	No	No
33	F	56	39	27	Yes	No	No

F, Female; M, male.
[a] Defined as two or more cancer foci.

alence of *BRAF* mutation in the primary PTC tumors was 63%, within the upper range reported in other studies (23). This prevalence is relatively high, reflecting the fact that all the cases in this series had lymph node metastasis and that *BRAF* mutation is generally reported to be associated with a higher prevalence of lymph node metastasis. Also, the PTC samples included in this study were all classical variants, which harbor *BRAF* mutation with a particularly high prevalence (23). As summarized in Table 1, 21 of these 33 cases harbored the T1799A *BRAF* mutation in their primary tumors. Seventeen (81%) of these 21 cases also harbored the same mutation in their metastasized tumor tissues in lymph nodes. The four cases of *BRAF* mutation-positive primary tumors that had no mutation in lymph nodes had multiple foci of PTC in the thyroid gland. It is therefore possible that in these four cases the lymph node-associated PTC tissues available to us for *BRAF* mutation analysis were metastasized from the PTC foci that were not examined and that might harbor no mutation. Figure 1 (*top*) shows a representative case (no. 15) with the T1799A *BRAF* mutation in both the primary tumor and the metastasized tumor in a lymph node. In one case (no. 5), we had two lymph nodes available for this study, and the *BRAF* mutation was found in the metastasized PTC tissues in both of the lymph nodes. Among the 12 cases that did not harbor *BRAF* mutation in the primary tumors, nine cases also did not harbor *BRAF* mutation in the metastasized tumor tissue in lymph nodes. Five of the latter nine cases had multifocal PTC in the thyroid gland, and therefore we were not certain whether the lymph node metastasis was from the primary tumor available to us for this study. The remaining four cases that did not harbor mutation either in the primary tumor or in the lymph node-metastasized tumor had only a solitary PTC without multifocality in the thyroid gland, suggesting that the *BRAF* mutation-negative lymph node metastasis was from the *BRAF* mutation-negative primary tumor examined in each of the four cases. Therefore, *BRAF* mutation is not absolutely required for metastasis of PTC to lymph nodes, consistent with clinicopathological studies that showed association of both *BRAF* mutation-positive and -negative primary PTC tumors with lymph node metastasis (2, 8–10, 12, 13). Interestingly, the other three cases (4, 16, and 25) that harbored no mutation in their primary tumors harbored the T1799A *BRAF* mutation in lymph node-metastasized tumor tissues. In one case (no. 4), a novel *BRAF* mutation, G1800A, was found in the metastasized tumor, which coexisted with the T1799A *BRAF* mutation, although the primary tumor harbored neither of the two mutations (Fig. 1, *bottom*). These two mutations form a tandem TG1799–1800AA mutation. Because careful pathological examination revealed only one PTC focus in the thyroid gland in each of the three cases, the mutations apparently developed *de novo* in the lymph node after metastasis occurred. The tandem TG1799–1800AA mutation was confirmed by sequencing the PCR products using the reverse primer (Fig. 1, *bottom*). When the PCR products of this case were subcloned, followed by sequencing, only two types of allelic patterns were seen, wild-type allele and the tandem TG1799–1800AA mutation, confirming a true tandem mutation within the same allele (Fig. 2). As a consequence of this tandem mutation, codon 600 (GTG) in the open reading

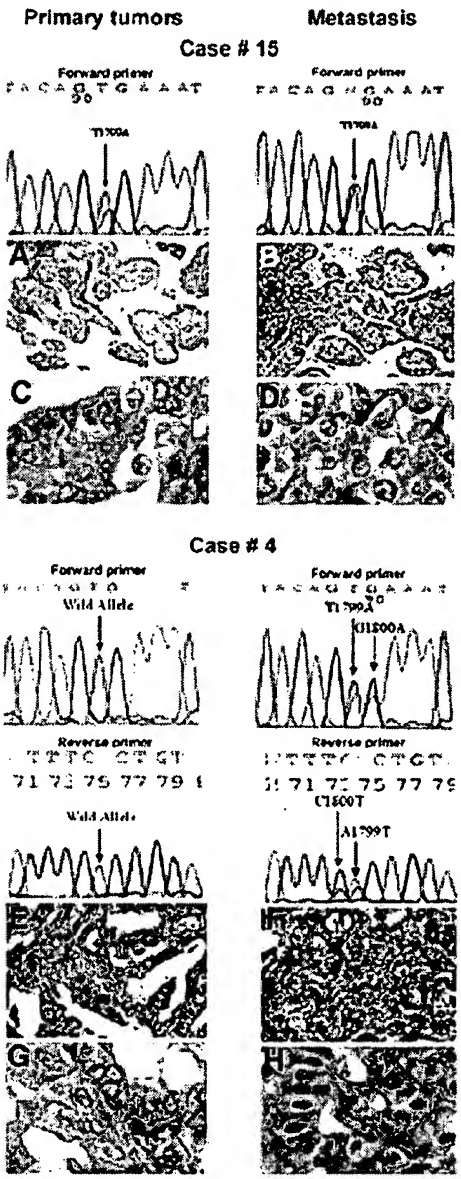

FIG. 1. *BRAF* mutations in primary PTC and matched lymph node-metastasized tumors. Shown in the *top* of the figure is a representative case (no. 15) that harbored the T1799A mutation in both the primary tumor (*left*) and the matched metastasized tumor tissue in a lymph node (*right*). A, The primary thyroid tumor showed typical papillary carcinoma architecture. C, The tumor cells demonstrated typical nuclear features for PTC: irregularity of nuclear contour, nuclear clearing, and pseudoinclusion. B and D, The lymph node metastasis demonstrated histological and cytological features similar to those observed in primary tumors. Shown in the *bottom* of the figure is a representative case (no. 4) that harbored no mutation in the primary tumor (*left*) but the T1799A mutation in the metastasized tumor tissue in a lymph node (*right*). Together with the T1799A mutation, a novel mutation, G1800A, was also found in the lymph node of this case, forming a tandem TG1799–17800AA mutation. E and G, Primary tumor of this case showed typical histological (E) and cytological (G) features for PTC. F, The metastasis demonstrated mixed follicular and papillary structures with predominance of follicular areas. H, In the areas of metastasis with papillary morphology, the neoplastic cells possessed typical nuclear features for PTC. In the follicular areas of the metastasis, the neoplastic cells had abundant eosinophilic cytoplasm and hyperchromatic nuclei (H), different from the nuclei in the primary tumor (G). For case 4, the tandem TG1779–1800AA *BRAF* mutations were confirmed by also sequencing the PCR products with the reverse primers (lower chromatogram of case 4).

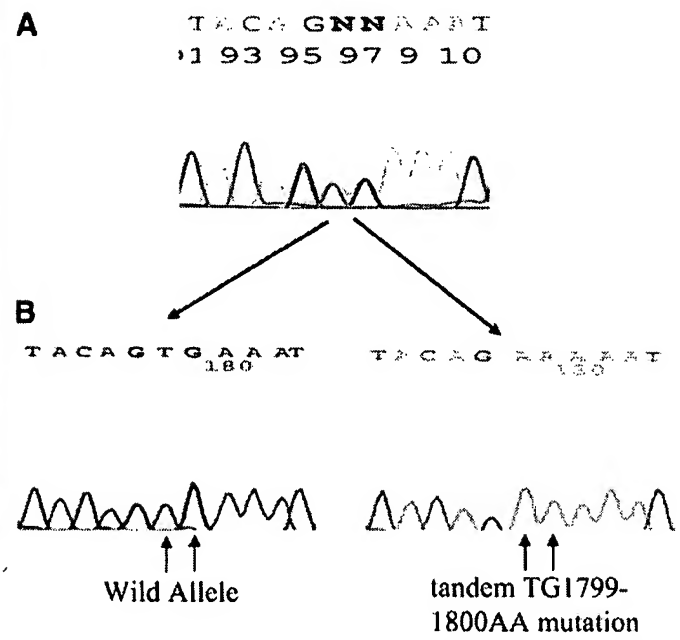

FIG. 2. Confirmation of the tandem TG1799–1800AA *BRAF* mutation within one allele by sequencing of subcloned *BRAF* PCR products. A, Direct DNA sequencing of the PCR products of *BRAF* exon 15, showing the tandem TG1779–1800AA mutation overlapped with the wild 1779–1800TG; B, DNA sequencing of the subcloned PCR products from A. Only clones with wild allele or clones with the tandem TG1799–1800AA mutation were identified, confirming a true tandem mutation within the same allele.

frame of the *BRAF* gene would be expected to be converted to GAA, resulting in a V600E (valine-to-glutamine) amino acid change in the BRAF protein, same as the amino acid change caused by the commonly seen T1799A *BRAF* mutation that converts codon 600 (GTG) to GAG. This tandem mutation in codon 600 would therefore be expected to confer the same oncogenic function to BRAF kinase as the T1799A mutation alone does. As a morphological representative for the cases that harbored the T1799A mutation in both the primary tumors and the matched lymph node-metastasized tumors, case 15 showed similar histological and cytological features consistent with PTC in both the primary and lymph node-metastasized tumors (Fig. 1, A–D). Interestingly, in case 4, which harbored the tandem TG1799–1800AA mutation in the lymph node-metastasized tumor but no mutation in the primary tumor, two morphologically distinct groups of tumor cells were present in the metastasized tumor tissue, one with typical nuclear features of PTC and the other with hyperchromatic nuclei and abundant eosinophilic cytoplasm (Fig. 1, E–H). The tandem TG1799–1800AA mutation has not been previously reported in thyroid cancers, although it was found in invasive melanomas (24).

Discussion

There have been extensive studies on the T1799A *BRAF* mutation in primary thyroid tumors (6–13). Significant association of this mutation in primary PTC tumors with metastasis and local invasion was shown in some studies (2, 9, 10) but not in others (8, 12, 13), although the studies in the latter group were relatively small. There have been no studies on *BRAF* mutation in metastasized thyroid tumors until recently (25). Demonstration of a correlation of *BRAF* mutation in primary and metastasized thyroid tumors would be consistent with a role of this mutation in facilitating metastasis of thyroid cancer.

The present study examined *BRAF* mutation in primary and matched lymph node-metastasized PTC tumors and represents a relatively large series of lymph node-metastasized PTC samples. We demonstrated a high prevalence of the T1799A mutation in metastasized thyroid tumors in lymph nodes whose primary tumors harbored the same mutation, suggesting that this mutation likely had been transferred with metastasis to lymph nodes from primary tumors. The finding of this mutation in both the primary tumor and the two matched lymph nodes in case 5 further supports this notion. It was interesting to find that some cases (4, 16, and 25) harbored the T1799A *BRAF* mutation only in the metastasized tissues in lymph nodes but not in their primary tumors. These cases harbored a solitary PTC tumor without multifocality in the thyroid gland, arguing against the possibility that the mutation in the lymph node examined might represent metastasis from a separate *BRAF* mutation-positive primary tumor. It is therefore possible that *BRAF* mutation may develop *de novo* in metastasized thyroid cancer cells in lymph nodes. The presence of the tandem TG1799–1800AA mutation only in lymph node metastasis but not in the primary PTC is also consistent with this possibility. Oler *et al.* (25) recently reported a novel mutation, a deletion of codon 601, only in lymph node-metastasized PTC but not in the primary tumors (25). It thus appears that lymph node predisposes the *BRAF* gene in thyroid cancer cells to the risk of being bombarded around the area of codons 600 and 601, resulting in genetic alterations. The high prevalence of *BRAF* mutation in lymph node-metastasized thyroid tumors in the cases that harbored this mutation in their primary tumors supports the notion that *BRAF* mutation may facilitate the seeding and progression of PTC cells in lymph nodes, consistent with the finding that *BRAF* mutation was associated with a higher prevalence of lymph node metastasis of PTC (2, 23).

The high prevalence and *de novo* formation of *BRAF* mutations in PTC metastasized to lymph nodes may suggest that lymph nodes possess a special local milieu that favors the survival and growth of *BRAF* mutation-positive thyroid cancer cells once they are seeded in the lymph node. The biological changes of thyroid cancer cells as a consequence of *BRAF* mutation, on the other hand, may confer thyroid cancer cells' unique ability to survive in the favorable local milieu of lymph nodes. In this model, *BRAF* mutation plays a role in the host-environment interaction in the process of PTC metastasis in the lymph node. This hypothesis is consistent with the common finding that follicular thyroid cancers, which do not harbor *BRAF* mutation, usually do not metastasize to lymph nodes (26), whereas PTC, which harbor *BRAF* mutation with a high prevalence, frequently metastasize to lymph nodes (21, 22). It may also explain a frequent phenomenon observed clinically that small primary micro PTC foci in the thyroid gland can be associated with large lymph node-metastasized PTC tumors (27, 28). It is likely that certain factor(s) in the local milieu of lymph node in-

teract specifically with *BRAF* mutation-positive thyroid cancer cells to facilitate their survival and promote their growth and progression in lymph nodes. When the selective pressure of the local milieu of the lymph node is strong enough, it may even cause *de novo* formation of *BRAF* mutation in metastasized PTC cells. It would be interesting to see whether certain growth factors in the local milieu of lymph nodes may play a role in host-tumor interactions between *BRAF* mutation-positive PTC and lymph modes as seen with IGF-I and epidermal growth factor released from lymph node stromal cells that can promote growth and tumorigenicity of breast carcinoma cells (29).

In summary, our study demonstrated a high prevalence of *BRAF* mutation in metastasized PTC in lymph nodes. We also showed possible *de novo* formation of the T1799A mutation and a TG1799–1800AA tandem mutation in codon 600 of the *BRAF* gene in metastasized PTC in lymph nodes. These results are consistent with the notion that *BRAF* mutation may facilitate the metastasis and progression of PTC in lymph nodes. We propose that the local milieu of lymph nodes may specially favor the selection and survival of thyroid cancer cells that harbor *BRAF* mutation. Additional work is needed to test this hypothesis.

Acknowledgments

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During the revision of this manuscript, a paper has been published by Oler *et al.* (25) reporting a high prevalence of *BRAF* mutation in lymph node metastases from PTC, similar to the finding in the present study, and a new lymph node metastasis-specific *BRAF* mutation that was not found in the present study.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	)	Confirmation No. 9884
David SIDRANSKY *et al.*	)	Group Art Unit: 1642
Application Serial No.: 10/821,203	)	Examiner: C.M. Joyce
Filed: April 8, 2004	)	
For: BRAF MUTATION T1796A IN THYROID CANCERS	)	Atty. Dkt No.: 001107.00463

DECLARATION UNDER RULE 132

I, David Sidransky, declare:

1. I am a named inventor on the subject application.

2. I am the director of the Head and Neck Cancer Research Division at Johns Hopkins University School of Medicine. In addition, I am professor of oncology, otolaryngology-head and neck surgery, cellular & molecular medicine, urology, genetics and pathology at John Hopkins University and Hospital. I am certified in internal medicine and medical oncology by the American Board of Medicine. My work has been published in more than 300 peer-reviewed publications, and I have contributed to more than 50 cancer reviews.

3. Based on literature-reported statistics for the incidence of cancer in the US and the incidence of T1796A BRAF mutations in cancers of the thyroid and cancers that metastasize to the thyroid, I believe that a blood test to detect a BRAF mutation in a person who is suspected of having a thyroid neoplasm would overwhelmingly detect thyroid tumors rather than BRAF mutations from tumors in other organs or even rarer metastases to the thyroid from other organs.

4. I base this conclusion on the following statistics and sources:

   a. Rate of thyroid cancer in suspicious thyroid nodules: 14.5 % [1]
   b. Rate of BRAF mutations in all thyroid cancers: 25 % [2]

[1] Tyler, *Annals of Surg. Onc.* 7: 376-3989, 2000

c. Rate of BRAF mutations in papillary thyroid cancers: 69 % [3]
d. Rate of BRAF mutations in lung cancers: 1 % [4]
e. Rate of BRAF mutations in colorectal cancers: 5 % [5]
f. Rate of BRAF mutations in melanoma cancers: 60 % [6]

g. U.S. population: approximately 301,465,095 [7]

h. Incidence cases of thyroid cancer in the US population: 30,180[8]
i. Incidence cases of lung cancer in the US population: 174,470 [9]
j. Incidence cases of colorectal cancer in the US population: 148,610[10]
k. Incidence cases of melanoma cancer in the US population: 62,190 [11]

l. Incidence cases of lung cancer with BRAF mutation in the US population: 1,744 (d x i)

m. Incidence cases of colorectal cancer with BRAF mutation in the US population: 7,430 (e x j)
n. Incidence cases of melanoma cancer with BRAF mutation in the US population: 37,314 (f x k)

o. Sum of incidence cases of lung, colorectal, and melanoma cancers in the US population with BRAF mutations: 46,488 (l + m + n)

p. The cancers other than thyroid that have BRAF mutations most frequently are lung, colon, and melanoma. [12] The total number of such cancers in the US is 385,270 (i + j + k).

5. Screening of the blood of unselected U.S. persons would yield a BRAF rate of 0.0154 % (o/g) due to lung cancer, colorectal cancer and melanoma.

6. Screening blood of U.S. patients selected for a suspicious nodule would yield a rate of 3.6 % (a x b).

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[2] Namba, J. *Clin. Endocrinol. Metab. 88*:4393-97, 2003
[3] Cohen, *J. Natl. Cancer Inst. 95*: 625-27, 2003
[4] Brose, *Cancer Research 62:* 6997-7000, 2002
[5] Wang, *Cancer Research 63:* 5209-5212, 2003
[6] Brose, *op. cit.*
[7] U.S. Census Bureau, http://www.census.gov/main/www/popclock.html
[8] Jemal *et al, CA Cancer J. Clin. " Cancer Statistics, 2006,"* 56: 106-130, at Table 1
[9] *Ibid.*
[10] *Ibid.*
[11] *Ibid.*
[12] Garnett and Marais, Cancer Cell "Guilty as charged: BRAF is a human oncogene," 6:313-319, 2004

7. If one screened 10,000 U.S. patients selected for a suspicious nodule one would expect to find that 360 of them had BRAF mutations due to a thyroid neoplasm and 15 of them had a BRAF mutation due to a lung, colon, or melanoma cancer.

8. Thus, if one were to conclude that a BRAF mutation in blood of a patient who had a suspicious thyroid neoplasm was due to a thyroid cancer, one would be correct 96 % of the time and one would be incorrect 4 % of the time.

9. Based on these data, I believe that detection of a BRAF mutation in blood of a patient suspected of having a thyroid neoplasm would be a useful technique.

10. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/27/07
Date

David Sidransky

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Confirmation No. 9884
David SIDRANSKY *et al.*	Group Art Unit: 1642
Application Serial No.: 10/821,203	Examiner: C.M. Joyce
Filed: April 8, 2004	
For: BRAF MUTATION T1796A IN THYROID CANCERS	Atty. Dkt No.: 001107.00463

DECLARATION UNDER RULE 131

We, David Sidransky, MingzhaoXing, and Yoram Cohen, declare:

1. We are the named inventors on the subject application.

2. Claims 1-5, 7, and 9 stand rejected as anticipated by or obvious over Kimura et al., "High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma," Cancer Res. 2003 Apr 1;63(7):1454-7.

3. The independent claim 1, upon which claims 2-5, 7, and 9 depend, is directed to a method for distinguishing malignant papillary from benign thyroid samples. The method comprises: determining presence of a T →A transversion at nucleotide 1796 of *BRAF* according to SEQ ID NO: 1 in a thyroid sample of a human. The presence of the transversion indicates a malignant papillary neoplasm and absence of the transversion indicates a benign neoplasm or sample.

4. We conceived and reduced the claimed invention to practice prior to April 1, 2003.

5. We conducted experiments, obtained data, wrote a manuscript describing the experiments and data, and submitted the manuscript to a scientific journal, all prior to April 1, 2003. One journal that we submitted a manuscript to is *Nature Genetics.* This journal archives its manuscript submissions and makes them available on-line to the original submitters. Attached as **Exhibit 1** is the letter which we sent to *Nature Genetics* requesting that they review our manuscript and publish it. The attached letter was downloaded from the *Nature Genetics* website. (The date has been redacted.) Attached as **Exhibit 2** is a copy of the manuscript that we submitted to *Nature Genetics.* The attached copy was downloaded from the *Nature Genetics* website. Attached as **Exhibit 3** is a copy of the index sheet from the *Nature Genetics* website, indicating the dates and disposition of the manuscript. (The dates have been redacted.) As evidenced by the index sheet the manuscript was never revised.

6. The content of the manuscript demonstrates that we had actually conceived of and reduced to practice a method comprising determining presence of a T →A transversion at nucleotide 1796 of *BRAF* according to SEQ ID NO: 1 in a thyroid sample of a human, wherein presence of the transversion indicates a malignant papillary neoplasm and absence of the transversion indicates a benign neoplasm or sample.

7. Specifically, the manuscript details testing 54 thyroid samples. Page 1, lines 17. Exon 15 of *BRAF* was amplified and then digested with endonuclease TsprI to identify *BRAF* T1796A mutations. Page 1, lines 19-20. Such mutations were identified in 60% of papillary thyroid carcinomas (page 2, lines 4-5) but in no benign thyroid conditions, follicular thyroid carcinomas, medullary thyroid carcinomas, or Hurthle cell carcinomas (page 2, lines 9-12). These data are also shown in Table 1 (page 5). An example of the experimental method and results is shown in Figure 1a.

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3/29/07	
Date	David Sidransky
Date	MingzhaoXing
Date	Yoram Cohen

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:	Confirmation No. 9884
David SIDRANSKY *et al.*	Group Art Unit: 1642
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Date	David Sidransky
Date 4/12/2007	MingzhaoXing
Date	Yoram Cohen